

Glycogen Synthase Kinase-3 β Phosphorylates Protein Tau and Rescues the Axonopathy in the Central Nervous System of Human Four-repeat Tau Transgenic Mice*

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Protein tau filaments in brain of patients suffering from Alzheimer's disease, frontotemporal dementia, and other tauopathies consist of protein tau that is hyperphosphorylated. The responsible kinases operating *in vivo* in neurons still need to be identified. Here we demonstrate that glycogen synthase kinase-3 β (GSK-3 β) is an effective kinase for protein tau in cerebral neurons *in vivo* in adult GSK-3 β and GSK-3 β \times human tau40 transgenic mice. Phosphorylated protein tau migrates slower during electrophoretic separation and is revealed by phosphorylation-dependent anti-tau antibodies in Western blot analysis. In addition, its capacity to bind to re-assembled paclitaxel (Taxol $^{\circledR}$)-stabilized microtubules is reduced, compared with protein tau isolated from mice not overexpressing GSK-3 β . Co-expression of GSK-3 β reduces the number of axonal dilations and alleviates the motoric impairment that was typical for single htau40 transgenic animals (Spittaels, K., Van den Haute, C., Van Dorpe, J., Bruynseels, K., Vandezande, K., Laenen, I., Geerts, H., Mercken, M., Scot, R., Van Lommel, A., Loos, R., and Van Leuven, F. (1999) *Am. J. Pathol.* 155, 2153–2165). Although more hyperphosphorylated protein tau is available, neither an increase in insoluble protein tau aggregates nor the presence of paired helical filaments or tangles was observed. These findings could have therapeutic implications in the field of neurodegeneration, as discussed.

Protein tau represents a family of neuronal phosphoproteins, which were originally identified as proteins that co-purify with

microtubules (MT)¹ and that assemble tubulin dimers into microtubules (1). Six low molecular weight isoforms are known, which contain none, one, or two N-terminal inserts of unknown function and three or four C-terminal repeat domains that are essential for binding to microtubules (2–4). The binding to microtubules involves both the protein tau repeat domains and some of the flanking regions (5, 6).

Phosphorylation of serine and threonine residues within or flanking these MT-interacting regions reduces the interaction with microtubules considerably (7–11). Among other kinases, GSK-3 β is identified as a potent proline-dependent protein tau kinase (12–15), also known as “tau protein kinase I” (16). GSK-3 β phosphorylates protein tau in cell-free systems (17) and in transfected cells, and thereby reduces its ability to initiate microtubule nucleation (18–23). Detailed biochemical and structural analyses demonstrate that GSK-3 β phosphorylates analogous epitopes on protein tau *in vitro* and in cell paradigms, as on protein tau embedded in paired helical filaments (PHF) of Alzheimer's disease patients (AD) (12). PHF are composed of hyperphosphorylated protein tau (24–28), and antibodies directed to GSK-3 β decorate PHF, indicating a direct association between GSK-3 β and hyperphosphorylated protein tau (29, 30). Moreover, active GSK-3 β accumulates in pre-tangle and tangle-bearing neurons in AD brain (31), and AD-related presenilin-1 clinical mutants are proposed to enhance the inter-reaction between protein tau and GSK-3 β (32). Unlike in Alzheimer's disease, in frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), dementia segregates with mutations in the tau gene. Intronic mutations and missense mutations S305N and N279K induce a preponderance of the four-repeat over the three-repeat isoforms of protein tau (33–35), and most clinical missense mutations located in the microtubule binding domains reduce the association of protein tau with microtubules (36–39). No matter through which mechanism (phosphorylation or mutation) the MT-binding and MT-stabilizing function of protein tau is compromised, filamentous intraneuronal inclusions consist of hyperphosphorylated protein tau in both dementing illnesses,

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¹ The abbreviations used are: MT, microtubule; GSK-3 β , glycogen synthase kinase-3 β ; WT, wild type; AD, Alzheimer's disease; PHF, paired helical filament; RAB, reassembly buffer; RIPA, radioimmunoprecipitation assay buffer; FA, formic acid; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; GFAP, glial fibrillary acidic protein; htau40-1, -2, and -5, transgenic mice of strain 1, 2, and 5, respectively, overexpressing human tau; htau40-1HH, homozygous mouse of strain htau40-1; TPK-I, tau protein kinase I.

in addition to other tauopathies.

Despite the wealth of *in vitro* data, convincing evidence for any functional repercussion of the phosphorylation of protein tau by GSK-3 β *in vivo* is lacking and the requirement of protein tau phosphorylation for PHF formation is still a matter of debate. We generated transgenic mice that overexpress a constitutively active form of the human kinase, *i.e.* GSK-3 β (S9A) with serine at position 9 replaced by alanine to prevent its inactivation by phosphorylation (40, 41). The GSK-3 β transgenic mice were crossed with transgenic mice that overexpress the longest isoform of human protein tau, *i.e.* containing 2 N-terminal inserts and 4 microtubule binding domains (htau40), extensively characterized previously (42). Both recombinant transgenic constructs were based on the adapted mouse *thy1* gene promoter, warranting co-expression specifically in neurons of the central nervous system (42–45).

Here we demonstrate that human GSK-3 β (S9A) hyperphosphorylates murine and human protein tau in the brain of single and double transgenic mice. The demonstrated phosphorylation of protein tau in the double transgenic mice reduces the binding of protein tau to isolated microtubules. Although more MT-unassociated protein tau is available, neither PHF nor tangles are formed and the amount of insoluble protein tau remains unaltered. Interestingly, protein tau hyperphosphorylation correlates with a strong reduction in the number of axonal dilations and with a nearly complete alleviation of the motoric problems observed in htau40 transgenic mice. A 2-fold increase in GSK-3 β activity, relative to the endogenous enzymatic activity, thereby rescues nearly all neuropathological symptoms of the single htau40 transgenic mice (42). The conclusion that hyperphosphorylation of protein tau by GSK-3 β reverses such a severe phenotype could have therapeutic implications in the field of neurodegeneration, as discussed.

MATERIALS AND METHODS

Generation of GSK-3 β Transgenic Mice—cDNA coding for human GSK-3 β (S9A) (Refs. 40 and 41; gift of J. Woodgett) was ligated in the adapted mouse *thy1* gene (42, 43) and was microinjected into 0.5-day-old FVB/N pronuclear mouse embryos. Transgenic founders were identified by Southern blotting and genotype of transgenic offspring, bred into the FVB/N genetic background, was performed on tail biopsy DNA by polymerase chain reaction. The htau40 transgenic mice have been described elsewhere (42). Three founder strains, *i.e.* htau40-1, htau40-2, and htau40-5, which transmitted the transgene in a stable Mendelian fashion, were used to generate double transgenic mice by cross-breeding with GSK-3 β (S9A) animals.

Sensorimotor Tests—Single and double transgenic mice at the age of 2–4 months were subjected to three classical sensorimotor tests (46), *i.e.* the forced swimming test, the rod walking test, and the inverted wire-grid test, performed as described previously (42). In addition, we scored the time that the mice needed to return upward after being forced on their back. Four groups of mice were tested: 21 wild-type FVB mice (WT), 23 homozygous htau40-1 (1HH), 17 heterozygous GSK-3 β (S9A) (GSK), and 7 htau40-1 \times GSK-3 β (S9A) double transgenic mice (1HH \times GSK). The contingency χ^2 test was used to evaluate the difference.

Tissue Extractions and Western Blotting—To prevent dephosphorylation during *post mortem* delay (47, 48) all tissues were rapidly dissected and tissue homogenates were processed on ice and immediately stored at -70°C after freezing in liquid nitrogen. Tissue extraction and Western blotting was performed as described previously (42). Reaction of the secondary antibody with mouse immunoglobulins in Western blotting was eliminated as described previously (42).

Phosphorylation-independent monoclonal antibodies against protein tau were HT-7 (Innogenetics, Gent, Belgium) and Tau-5 (PharMingen, San Diego, CA). Monoclonal antibodies directed against phosphorylated protein tau epitopes were AT-8, AT-180 (Innogenetics, Gent, Belgium), PHF-1 (P. Davies, New York), AD-2 (B. Pau, Lille, France), and 12E8 (P. Seubert, Élan Pharmaceuticals, South San Francisco, CA). Their respective epitopes are: Ser(P)-199 and Ser(P)-202 (49, 50), Thr(P)-231 (51), Ser(P)-396 and/or Ser(P)-404 (PHF-1, see Ref. 52; AD-2, see Ref. 53), Ser(P)-262 and Ser(P)-356 (54). Monoclonal antibody Tau-1 (Roche

Molecular Biochemicals, Mannheim, Germany) recognizes an epitope comprising non-phosphorylated Ser-199 and Ser-202 (50).

Dephosphorylation of protein tau in brain homogenates by pretreatment with alkaline phosphatase was performed as described (42).

The solubility of protein tau in brain and spinal cord from wild type, single, and double transgenic mice was determined by sequential extraction with high salt reassembly buffer (RAB), detergents containing radioimmunoprecipitation assay buffer (RIPA) and 70% formic acid (FA), as described (55). Four htau40-1 and htau40-1 \times GSK-3 β transgenic mice were used, quantitative Western blot analysis was carried out using Tau-5 antibody and the amount of protein tau in the different buffers was determined, with the total transgenic human protein tau taken as 100%.

GSK-3 β Protein and Enzymatic Activity—The GSK-3 β (S9A) protein levels in brain and spinal cord extracts were estimated by Western blotting with the monoclonal antibody TPK-I/GSK-3 β (Affinity, Nottingham, United Kingdom). The GSK-3 β enzymatic activity was measured in brain homogenates after immunoprecipitation and fractionation by cation exchange fast protein liquid chromatography on a Mono S column (Amersham Pharmacia Biotech, Uppsala, Sweden) as described (56).

Quantification of Axonopathy in the Central Nervous System—Non-transgenic ($n = 4$), GSK-3 β (S9A) ($n = 4$), htau40-1 ($n = 8$), htau40-2 ($n = 8$), htau40-1 \times GSK ($n = 4$), and htau40-2 \times GSK ($n = 4$) mice were transcardially perfused with paraformaldehyde (4% in phosphate-buffered saline). Brain and spinal cord were immersion-fixed overnight, and vibratome (40 μm) and microtome (6 μm) sections were cut and processed (42). Axonal dilations were detected and quantified as described (42). The Kruskal-Wallis test was applied to evaluate the differences.

Binding of Protein Tau to Isolated Microtubules—Paclitaxel-dependent isolation and re-assembly of microtubules and isolation of microtubule-associated proteins from brain and spinal cord was performed as described (57). To increase the yield of microtubule preparations from mouse spinal cord, 3 mM CaCl_2 was additionally added to the MT buffer to depolymerize cold-stable microtubules *in situ* (58). Equal amounts of proteins were loaded on 8% SDS-polyacrylamide gels after removal of mouse immunoglobulins. Densitometric quantification of Western blots of protein tau was as described (42), and results were normalized for tubulin and Tau-5. The blots for tubulin were developed with an antibody specific for neuronal β -III anti-N-tubulin (Promega, Madison, WI). The Wilcoxon signed-rank test was used to evaluate the differences.

Histochemistry and Immunohistochemistry—Mice were anesthetized and transcardially perfused with paraformaldehyde (4% in phosphate-buffered saline). Brain and spinal cord were immersion-fixed overnight and cut sagittally into two hemispheres or transversally into four tissue blocks of 9 mm, respectively. Vibratome sections (40 μm) were cut from the right hemisphere, whereas the left hemisphere was dehydrated, embedded in paraffin, and used for microtome sectioning (6 μm). Likewise, vibratome sections were cut from the thoracic part of the spinal cord, whereas the thoracolumbar part was embedded in paraffin. Primary antibodies used were monoclonal antibodies for protein tau (HT-7) and for GSK-3 β (TPK-I) (Affinity), a polyclonal antiserum against synaptophysin (Dako, Glostrup, Denmark), the monoclonal antibody SMI-32 for detection of neurofilament proteins (Affinity), and an antiserum to GFAP (Dako). Other procedures were performed as described: standard hematoxylin/eosin staining for muscle and Bielschowsky's silver impregnation and thioflavine S staining for central nervous system (42, 44).

Ultrastructural Analysis—For transmission electron microscopy, 4–8-month-old htau40-1HH ($n = 8$), htau40-1HH \times GSK-3 β ($n = 4$), and wild-type ($n = 4$) mice were perfused with 4% glutaraldehyde in phosphate-buffered saline or 4% paraformaldehyde and 0.1% glutaraldehyde. Areas of neocortex, hippocampus, and spinal cord were excised from 40- μm -thick vibratome sections, postfixed with OsO_4 , and embedded in epon.

For immunoelectron microscopy areas of cerebral neocortex, hippocampus, thalamus and subiculum were excised from 40- μm -thick vibratome sections of 6-month-old htau40-1HH ($n = 4$) and htau40-1HH \times GSK-3 β ($n = 2$) mice and processed as described (59). The following antibodies were used: B19 (anti-polyclonal; gift of J. P. Brion, Free University of Brussels, Belgium), anti-Tau (polyclonal; BioMakor, Rehovot, Israel), and AT8 (monoclonal; Innogenetics, Gent, Belgium) against protein tau; and anti-GFAP (polyclonal; Dako, Glostrup, Denmark). Fragments of hippocampus from an Alzheimer's disease patient, processed in the same way, were used as a positive control for tangle detection.

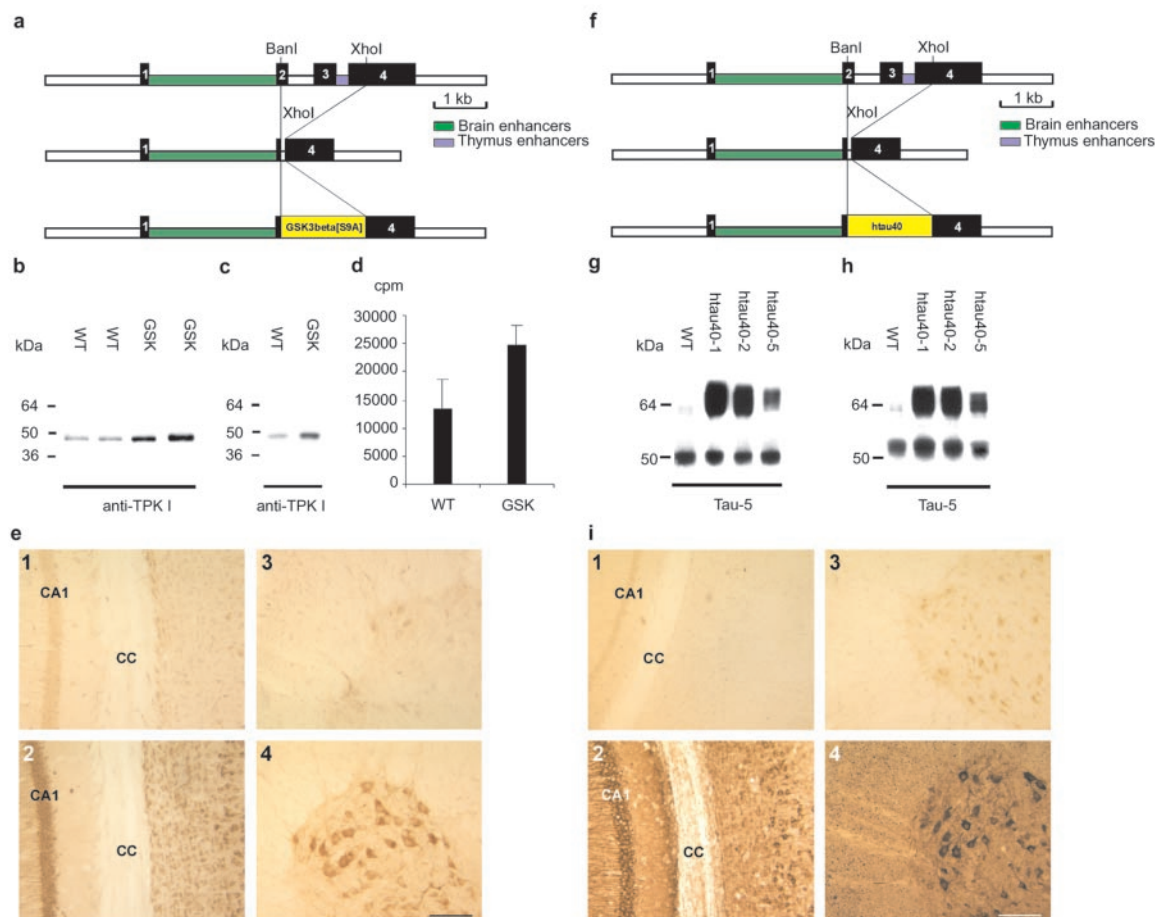


FIG. 1. Generation and primary characterization of GSK-3 β (S9A) and httau40 transgenic mice. *a* and *f*, structure of the mouse *thy1* gene and the recombinant DNA constructs. Original *thy1* gene exons (black boxes) are numbered. *b* and *c*, Western blotting with antibody GSK-3 β /TPK-I of extracts of brain (*b*) and spinal cord (*c*) from non-transgenic and heterozygous GSK-3 β transgenic mice. *d*, increased GSK-3 β kinase enzymatic activity in brain homogenates of GSK-3 β transgenic mice toward the GS-1 peptide ($p < 0.01$). Data are mean with S.D. of four independent determinations. *e*, immunohistochemical localization of GSK-3 β in the corticohippocampal region (panels 1 and 2), and ventral horn and adjacent white matter (panels 3 and 4) in double transgenic (panels 2 and 4) and non-transgenic (panels 1 and 3) mice. *g* and *h*, Western blotting with antibody Tau-5 of extracts of brain (*g*) and spinal cord (*h*) from non-transgenic and transgenic httau40-1 mice. The upper bands represent the human tau40 isoform; the lower band is murine protein tau. *i*, immunohistochemical localization of human protein tau with antibody HT-7, in the corticohippocampal region (panels 1 and 2), ventral horn and adjacent white matter (panels 3 and 4) in httau40-1 \times GSK-3 β double transgenic (panels 2 and 4) and non-transgenic (panels 1 and 3) mice. CC, corpus callosum; CA1, region of the hippocampus. Bars, 100 μ m.

RESULTS

Generation of Single GSK-3 β (S9A) and Double GSK-3 β (S9A) \times hTau40 Transgenic Mice—We have generated transgenic mice that express a mutant form of human GSK-3 β , denoted GSK-3 β (S9A), containing alanine in position 9 instead of the wild-type serine, to prevent inactivation by phosphorylation (60). The cDNA was incorporated in a recombinant DNA construct based on the mouse *thy1* gene promoter (Fig. 1*a*), and transgenic mice were generated by micro-injection, in the FVB mouse strain (42–44).

The human GSK-3 β protein was demonstrated by Western blotting in brain and spinal cord (Fig. 1, *b* and *c*). The transgene was enzymatically active on a synthetic peptide substrate, resulting in a doubling of the total GSK-3 β kinase activity in GSK-3 β mouse brain homogenates, relative to wild-type mice (Fig. 1*d*).

Transgenic mice that overexpress the longest human protein tau isoform (httau40) (Fig. 1, *f*–*h*) were characterized extensively (42). Double transgenic mice, heterozygous for each transgene, were obtained by cross-breeding.

Immunohistochemically, the human transgenic proteins were expressed exclusively in neurons, with high expression in the cortex and hippocampus. In addition, we observed that neurons in the spinal cord also expressed human GSK-3 β (S9A)

and human protein tau (Fig. 1, *e* and *i*). Both transgenes were thus expressed in the same cells of brain and spinal cord.

GSK-3 β Phosphorylates Murine and Human Protein Tau in Vivo—A panel of antibodies known to recognize epitopes on protein tau that are phosphorylated by GSK-3 β (61) were used for Western blot analysis of brain homogenates of transgenic mice. Murine protein tau was not more phosphorylated in young GSK-3 β (S9A) transgenic mice (4–8 weeks) compared with age-matched wild type mice (data not shown). In brain of older GSK-3 β transgenic mice (6–7 months), however, slower migrating protein tau isoforms reacted with phosphorylation-dependent antibodies AT-8 and AT-180, which were absent in brain of wild type mice. In addition, protein tau isoforms with slower electrophoretic mobility were also demonstrated with antibodies Tau-5 and PHF-1, only in brain of GSK-3 β transgenic animals (Fig. 2*a*). On the other hand, human protein tau was already hyperphosphorylated in brain extracts of young (5 weeks) double httau40-1 and httau40-5 \times GSK-3 β transgenic mice. Antibodies AT-8, AT-180, and AD-2 reacted with slow migrating human protein tau isoforms, and these were virtually absent in brain extracts of single transgenic littermates (Fig. 2*b*). Dephosphorylation of protein tau by pretreatment with alkaline phosphatase of brain extracts of single and double transgenic mice prior to electrophoresis, yielded identical

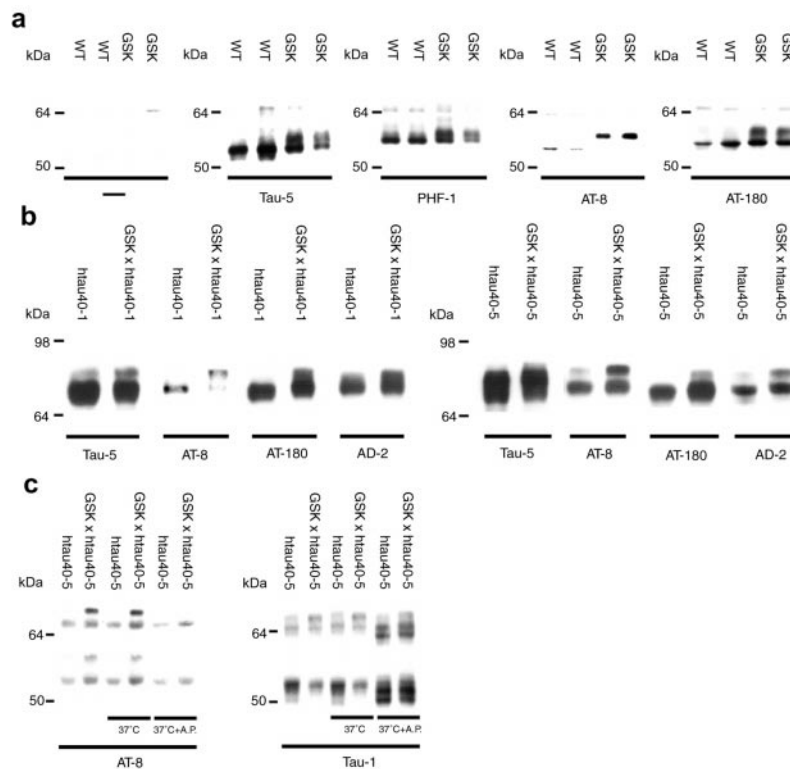


FIG. 2. Protein tau is hyperphosphorylated in brain of GSK-3 β (S9A) transgenic mice. *a* and *b*, Western blotting of brain extracts of 7-month-old single GSK-3 β (S9A) (*a*) and 5-week-old double GSK-3 β (S9A) \times htau40 (*b*) transgenic mice. In *a*, each panel compares brain extracts from two WT and two GSK-3 β (S9A) transgenic (GSK) mice. Immunoblots demonstrate retardation in electrophoretic mobility (Tau-5 and PHF-1) and increased immunoreaction with phosphate-dependent antibodies (AT-8 and AT-180) of phosphorylated protein tau. Brain homogenates were purified from mouse IgG prior to electrophoresis, as demonstrated by incubation of the blot with only secondary antibody (-). *b*, brain extracts from htau40-1 and htau40-5 transgenic mice and the respective double htau40 \times GSK-3 β (S9A) transgenic littermates show increased phosphorylation of human protein tau when immunoblotted with the specified monoclonal antibodies. Compared with murine protein tau, human protein tau requires much shorter exposure times when incubated with phosphate-dependent protein tau antibodies. Therefore, only the human protein is shown in these Western blots. *c*, brain homogenates of single and double htau40-5 and htau40-5 \times GSK-3 β (S9A) transgenic mice were applied either untreated, or after incubation at 37°C without or with alkaline phosphatase prior to Western blotting. Decreased AT-8 immunoreactivity and elimination of the slow migrating protein tau isoforms after alkaline phosphatase treatment demonstrate that human and mouse protein tau is extra phosphorylated in brain of GSK-3 β transgenic mice. Increased Tau-1 immunoreactivity illustrates that alkaline phosphatase treatment effectively dephosphorylates human and mouse protein tau. The remaining AT-8 signal is due to incomplete dephosphorylation of protein tau since alkaline phosphatase incubation time was restricted to prevent protein degradation when incubated at 37°C.

protein tau patterns on Western blotting with antibody Tau-1, and reduced AT-8 immunoreactivity of murine and human protein tau (Fig. 2*c*). In addition, dephosphorylation of protein tau resulted in more distinct bands (Fig. 2*c*, *Tau-1* panel).

To determine if hyperphosphorylation by GSK-3 β affected protein tau binding to microtubules, we examined its binding to paclitaxel-mediated reassembled MT. The amount of protein tau bound to MT was significantly reduced in preparations derived from brain and spinal cord of htau40-1 \times GSK-3 β double transgenic mice, compared with htau40-1 littermates (Fig. 3). Addition of lithium in all buffers during the isolation and reassembly process did not affect the reduced binding of protein tau to the microtubules. Thus, the reduced binding was not due to phosphorylation of protein tau by GSK-3 β *in vitro* during the isolation and assembling procedure (62), but occurred *in vivo* (Fig. 3, *a-f*). The soluble protein tau that remained in the supernatant after microtubule assembly and isolation was also hyperphosphorylated, as demonstrated by reaction with antibodies Tau-1, AT-180, and AD-2. Semiquantitative estimations identified the epitope recognized by AD-2 and PHF-1 to be most abundant (Fig. 4*a*). Quantification by densitometric scanning and normalization to reaction with antibody Tau-5 demonstrated a 4-fold increase of phosphorylation at the epitope defined by AD-2 (Fig. 4*b*). Antibodies AT-180 and Tau-1 revealed a more moderate increase in the phosphorylation of their respective epitopes in protein tau in the double

transgenic mice, relative to the single htau40 transgenic mice.

As phosphorylation at the AD-2 epitope is significantly increased in brain of GSK-3 β transgenic mice, its effect on the MT-binding capacity of protein tau was assessed. The amount of MT-associated human protein tau that was phosphorylated at the AD-2 epitope was only about half of the amount in the MT-unbound fraction, relative to the total amount of human protein tau in each fraction separately, detected by the Tau-5 antibody. Even more prominent was the at least 4-fold reduction in the amount of murine protein tau phosphorylated at the AD-2 epitope that bound to microtubules (Fig. 4*c*).

These data demonstrate that both murine and human protein tau are hyperphosphorylated in brain of GSK-3 β transgenic mice and that their ability to associate with microtubules is thereby reduced.

Absence of Paired Helical Filament Formation in GSK-3 β \times hTau40 Transgenic Mice—Bielschowsky's silver impregnation and thioflavine S staining (data not shown) did not reveal the presence of tangles or protein tau filaments in central nervous system of double transgenic mice (Figs. 5 and 6). Ultrastructurally, polyclonal antibodies to protein tau strongly labeled the paired helical filaments in the hippocampus of the Alzheimer's patient. In the transgenic htau40-1 and htau40-1 \times GSK-3 β mice, however, these antibodies reacted with protein tau in cell bodies, dendrites, axons, and dilated axons, but reactivity was much weaker than with paired helical filaments

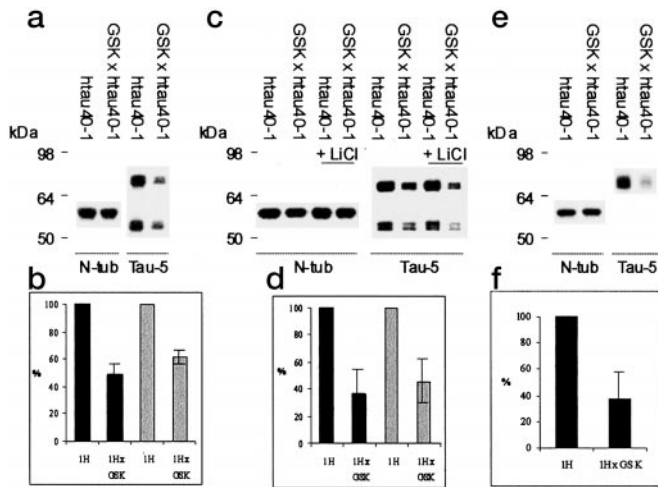


FIG. 3. Determination of protein tau bound to paclitaxel-stabilized microtubules. *a*, Western blots of tubulin and microtubule-associated human and murine protein tau in paclitaxel-stabilized microtubule pellets from brain of single and double transgenic mice. Tubulin and protein tau was determined by immunoblotting with neuron-specific β -III anti-N-tubulin and Tau-5 antibody, respectively. *b*, quantification by densitometric scanning of the amount of associated protein tau/tubulin for each mouse individually, normalized to htau40-1 transgenic mice ($n = 5$). *c*, Western blotting as in *a* with LiCl (10 mM) added during the *in vitro* assembly of microtubules. *d*, quantification of the LiCl-treated samples of panel *c* as in *b* ($n = 3$). *e*, Western blotting as in *a* of spinal cord of single and double transgenic mice. Even though the yield of re-assembled microtubules and associated protein tau derived from spinal cord was improved (see "Materials and Methods"), murine protein tau remained undetectable. *f*, quantification of Western blots displayed in *e* ($n = 3$). The reduction in amount of protein tau associated with microtubules in the central nervous system of double compared with single transgenic mice is significant, both in LiCl-treated ($p < 0.001$) and untreated ($p < 0.05$) conditions. *Panels a, c, and e* are representative experiments. *Black and gray boxes* represent human protein tau/tubulin and murine protein tau/tubulin, respectively. *Error bars* represent S.E. *IH*, heterozygous htau40-1 mouse; *n*, number of htau40-htau40 \times GSK-3 β couples used. All mice were older than 4 months.

in AD brain. Paired helical or straight filaments similar to those in Alzheimer's patients or other diseases with protein tau pathology were not present (Fig. 5, *a-d*). In addition, monoclonal antibody AT8 reacted with filamentous protein tau in the Alzheimer's disease patient, but no AT8 reactive filaments were present in brain tissue from transgenic mice (data not shown).

The solubility of protein tau in brain and spinal cord of htau40-1 and htau40-1 \times GSK-3 β transgenic 7-month-old mice was compared by differential extraction (see "Materials and Methods"). Although the majority of protein tau is soluble in the high salt RAB, a substantial amount of RAB-insoluble transgenic protein tau was present in the RIPA-soluble fraction, and even in the FA-soluble fraction. The amount of RIPA-soluble (htau40-1 = $23 \pm 1.9\%$; htau40-1 \times GSK-3 β = $19 \pm 1.7\%$) and FA-soluble (htau40-1 = $1.25 \pm 0.14\%$; htau40-1 \times GSK-3 β = $1.12 \pm 0.20\%$) protein tau was similar ($p > 0.05$) in brain of double compared with single htau40 transgenic animals. Similarly, in spinal cord, the amount of human protein tau dissolved in the RIPA (htau40-1 = $17 \pm 0.9\%$; htau40-1 \times GSK-3 β = $16 \pm 0.9\%$) and FA buffer (htau40-1 = $2.5 \pm 0.20\%$; htau40-1 \times GSK-3 β = $2.3 \pm 0.15\%$) was not elevated in htau40-1 \times GSK-3 β transgenic mice (Fig. 5*e*).

GSK-3 β Rescues the Axonopathy and Motoric Impairment of hTau40 Transgenic Mice—Transgenic mice that overexpress human protein tau at moderate levels have no severe phenotype (63, 64), whereas considerable overexpression of protein tau results in transgenic mice that develop axonopathy and

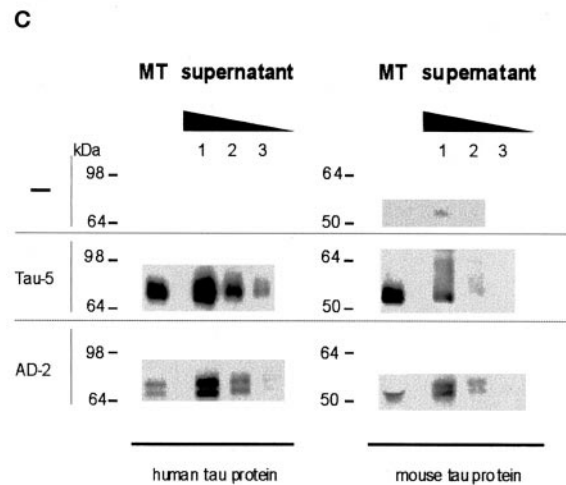
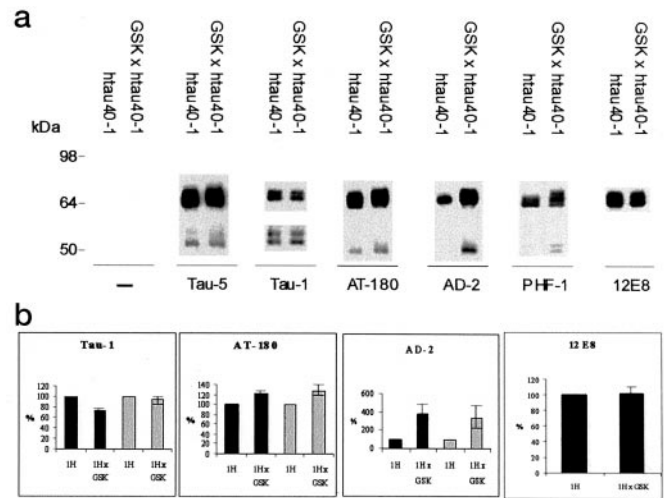


FIG. 4. Determination of protein tau phosphorylation when unassociated with paclitaxel-stabilized microtubules, and distribution of protein tau isoforms phosphorylated at the AD-2 epitope between MT-bound and unbound fraction. *a*, Western blots of unbound protein tau with antibodies indicated. *b*, quantification of unbound protein tau detected by the respective antibodies, normalized to the appropriate reaction with Tau-5 for each mouse individually, and relative to htau40-1 mice ($n = 4$). Differences are significant for AD-2 ($p < 0.001$), AT-180 ($p < 0.05$), and Tau-1 ($p < 0.05$). *Black and gray boxes* represent human and murine protein tau, respectively. *c*, distribution of the phospho-epitope defined by antibody AD-2 on protein tau bound to (MT) or un-associated with (supernatant) microtubules, derived from htau40-1 \times GSK-3 β transgenic mice ($n = 4$). Three (1–3; 13, 6.5, 3.25 μ g, respectively) dilutions of the corresponding supernatant were applied. *Panels a and c* are representative experiments. *Error bars* represent S.E. *IH*, heterozygous htau40-1 mouse; $-$, blots solely incubated with secondary antibody; *n*, number of htau40-htau40 \times GSK-3 β couples used. All mice were older than 4 months.

motoric problems (42, 55, 65).

In brain and spinal cord of single htau40 transgenic mice, Bielschowsky's silver impregnation, anti-neurofilament, and anti-synaptophysin immunohistochemical stainings revealed dilated axons, the number of which was reduced when transgenic GSK-3 β was coexpressed (Figs. 6 and 7, Table I). This was observed for two different parental mouse strains (htau40-1 and htau40-2), transgenic for human protein tau at different expression levels (Table I) (42). Moreover, the disorganization of the microtubule cytoskeleton was reversed in the central nervous system of double transgenic mice compared with single htau40 transgenic animals. In a number of dilated axons of htau40 single transgenic mice, the cytoskeleton was disrupted and numerous microtubules, randomly oriented, engirdled accumulations of pleomorphic vesicles, dense-cored ves-

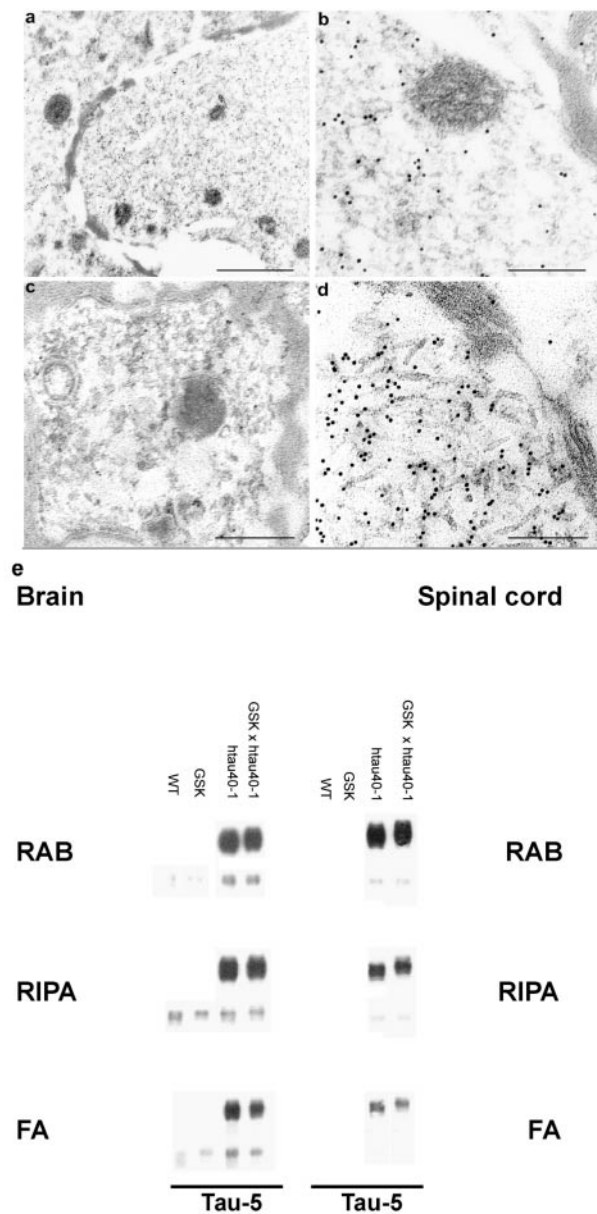


FIG. 5. Immunogold labeling of brain and biochemical determination of protein tau solubility in brain and spinal cord of single and double transgenic mice. *a*, immunogold staining with polyclonal antibody B19 of a dilated axon in the hippocampus of a 6-month-old httau40-1 HH mouse. Note that the gold particles are equally dispersed in the axoplasm. *b*, higher magnification of a dilated axon. *c*, nondilated axon of a 6-month-old httau40-1HH × GSK-3 β mouse showing rare gold particles in the axoplasm. *d*, paired helical filaments in a dystrophic neurite in the hippocampus of an Alzheimer's disease patient. Compared with *panel b*, the density of gold particles is much higher over the paired helical filaments. *Bars* measure 550 nm (*a*), 200 nm (*b* and *d*), and 375 nm (*e*). Determination of solubility of transgenic human protein tau in brain and spinal cord of wild type mice and GSK-3 β , httau40-1, and httau40-1 × GSK-3 β heterozygous transgenic littermates of 7 months old. Both tissues were sequentially extracted with RAB buffer, RIPA buffer, and FA. The amount of RAB-insoluble protein tau represented in the RIPA and FA fractions did not increase in GSK-3 β and httau40-1 × GSK-3 β mice compared with wild type and httau40-1 littermates, respectively.

icles, and smooth endoplasmic reticulum. In the dilated axons, the amount of microtubules was high relative to the number of neurofilaments, whereas in the unaffected axons of double transgenic and wild type mice the cytoskeleton was mainly composed of neurofilaments (Fig. 7, *g-j*). Concomitantly, the grouping of atrophic fibers and the fascicular atrophy in single httau40 transgenic mice, diagnostic for neurogenic

atrophy, was dramatically reduced in the double transgenic mice. The quadriceps of double transgenic mice was completely normal and devoid of any muscle wasting (Fig. 6, *e* and *f*).

We previously demonstrated that the severity of the axonopathy and motoric problems in httau40 mice were closely related (42). The effect of co-expression of GSK-3 β on the motoric phenotype was evaluated by four different tests on httau40-1 HH × GSK-3 β double transgenic mice, relative to single transgenic animals expressing either protein tau (httau40-1 HH) or GSK-3 β (S9A), and relative to wild-type mice (Fig. 8).

Overall, the double transgenic mice behaved in all tests significantly better than their single parental strains, with the exception of the rod-walking test. In the "uprighting reflex," the impairment of the single httau40 transgenic mice was nearly completely corrected in the double transgenic mice by co-expression of GSK-3 β (Fig. 8*a*). In the forced swimming test and inverted grid-hang test, the double transgenic mice performed equally well as wild-type mice and significantly better than single httau40 or GSK-3 β transgenic mice (Fig. 8, *b* and *c*). The number of mice that were unable to walk on the rod, however, did not differ between single httau40 and double transgenic mice, demonstrating that all httau40 transgenic mice were unable to remain on the rod, a characteristic not affected by GSK-3 β co-expression (data not shown). Interestingly, increased GSK-3 β activity itself negatively affected motoric capacities in the swimming and grid-hanging test when compared with wild type mice, but co-expression with human protein tau rescued to a large extent or even completely, the phenotype of GSK-3 β transgenic mice.

DISCUSSION

The kinases that phosphorylate protein tau *in vivo* have not been identified yet. The hypothesis that GSK-3 β is such a kinase was tested here experimentally by generating transgenic mice that overexpress in the central nervous system a constitutively active kinase, *i.e.* GSK-3 β (S9A). Obvious overexpression was obtained, resulting in a 2-fold increase of GSK-3 β kinase activity. In depth characterization of the GSK-3 β transgenic mice is still ongoing, but no overt pathology has been observed.

Experimental *in vivo* evidence is limited to a recent report describing somewhat augmented phosphorylation of mouse protein tau in brain of 18-month-old GSK-3 β transgenic mice (66). Indirectly, phosphorylation of protein tau at the Tau-1/AT-8 site was suggested to be dynamically regulated by GSK-3 β after stimulation of adult neuronal progenitor cells from adult rat hippocampus by fibroblast growth factor-2 (67).

In our study, the ability of GSK-3 β to phosphorylate protein tau was evident in GSK-3 β transgenic mice, and even more so in double transgenic mice, generated by cross-breeding with transgenic mice that overexpress the longest isoform of human protein tau, characterized previously (42). Hyperphosphorylation was apparent from slower migrating isoforms of protein tau, which reacted with specified monoclonal antibodies in Western blotting, such as AT-8, AT-180, and AD-2. These antibodies define phosphorylated epitopes on protein tau that are generated by GSK-3 β *in vitro*. In single GSK-3 β transgenic mice, phosphorylation of the endogenous murine protein tau became evident relative late, *i.e.* not before the age of 5–7 months. Hyperphosphorylation of human protein tau, on the other hand, was already evident in brain of young double transgenic mice, *i.e.* at the age of 4–6 weeks. Several factors could be responsible for this marked age difference, including different antibody affinity for mouse and human protein tau.

Binding of protein tau to microtubules is supposed to be regulated by phosphorylation. The amount of protein tau asso-

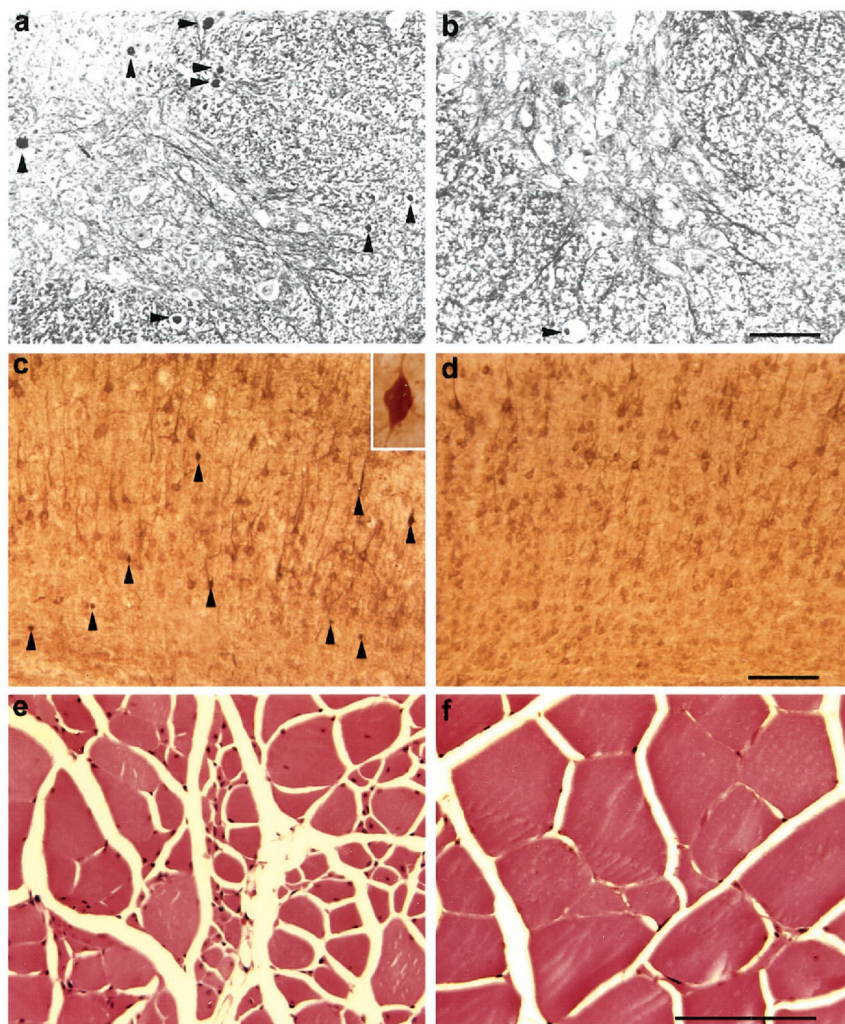


FIG. 6. Comparison of central nervous system and quadriceps skeletal muscle from homozygous htau40-1 (1HH) and htau40-1 (1HH) \times GSK-3 β double transgenic mice. Panels *a*, *c*, and *e* are from htau40 transgenic mice; panels *b*, *d*, and *f* from htau40 \times GSK-3 β double transgenic mice. *a* and *b*, reduced number of argyrophilic dilated axons in the gray matter of the ventral horn and surrounding white matter (arrowheads) in double compared with single htau40 transgenic mice. *c* and *d*, low power view of the neocortex stained with monoclonal antibody SMI-32, showing dilated axons (arrowheads) only in the cortex of single htau40 transgenic mice. Inset in *c* displays a higher magnification of a dilated axonal segment. *e* and *f*, hematoxylin/eosin staining of quadriceps muscle. Atrophic fibers are absent in quadriceps of double transgenic mice. Bars, 100 μ m. Mice are 2–4 months old.

TABLE I
Quantification of dilated axons in brain and spinal cord of heterozygous transgenic mice

Numbers of dilated axons in the spinal cord and cerebral neocortex are reduced by about an order of magnitude in mice coexpressing GSK-3 β . All differences between single and double transgenic mice are significant ($p < 0.05$). Mice used were 3 months of age. *n*, number of mice analyzed.

Mouse	Spinal cord		Cerebral cortex	
	<i>n</i>	No. dilated axons (\pm S.E.)	<i>n</i>	No. dilated axons (\pm S.E.)
WT	4	0	4	0
GSK	4	0	4	0
hTau40-1	8	9.9 \pm 0.49	8	10.7 \pm 1.15
hTau40-1 \times GSK	4	2.2 \pm 0.34	4	0.5 \pm 0.08
hTau40-2	8	3.6 \pm 0.84	8	2.6 \pm 0.21
hTau40-2 \times GSK	4	0.3 \pm 0.07	4	0.3 \pm 0.05

ciated with microtubules was reduced by 50% in preparations from brain and spinal cord of double transgenic mice compared with single htau40 transgenic animals. Moreover, unbound protein tau was hyperphosphorylated. Especially at the AD-2 epitope, free protein tau from brain of double transgenic mice was even more phosphorylated, exceeding the phosphorylation level of unbound protein tau of single htau40 transgenic mice about 4 times. In addition, the amount of protein tau isoforms phosphorylated at the AD-2 epitope was enriched in the unbound protein tau fraction at the expense of protein tau bound to the microtubules. Hence, phosphorylation at the AD-2 epitope is directly or indirectly, *i.e.* in concert with other phos-

phorylated residues recognized by antibodies AT-8 and AT-180, related to the reduced binding of protein tau to microtubules. However, since AD-2 immunoreactive isoforms are still detectable when bound to microtubules, the phosphorylation of this epitope does not eliminate the binding of protein tau to microtubules, but contributes negatively to this binding. The phosphorylation of the AD-2 epitope involves serine 396 and 404 in protein tau. These are thereby identified as essential determinants governing protein tau-microtubule interaction *in vivo*, corroborating studies in transfected cells (9). Also in extracts of brain biopsies from AD patients, protein tau phosphorylated at serines 396 and 404 was significantly hampered in binding to microtubules (54). Our findings are the *in vivo* correlate of the reduced binding of protein tau to microtubules in NT2N cells, transfected with GSK-3 β (68), and corroborate the capacity of GSK-3 β to hyperphosphorylate protein tau in neurons in the central nervous system of mice.

As hyperphosphorylation of protein tau might be an upstream phenomenon of PHF formation or even a prerequisite for filament formation, we scrutinized the central nervous system of transgenic mice for tangle-like structures. Neither Bielschowsky's silver impregnation, nor thioflavine S staining, nor ultrastructural analysis, nor immunogold labeling revealed signs of neurofibrillary tangles or protein tau filamentous aggregates in brain and spinal cord of single or double transgenic mice. Moreover, although more unbound protein tau is available, the amount of insoluble protein tau was not increased in brain and spinal cord of double transgenic mice compared with

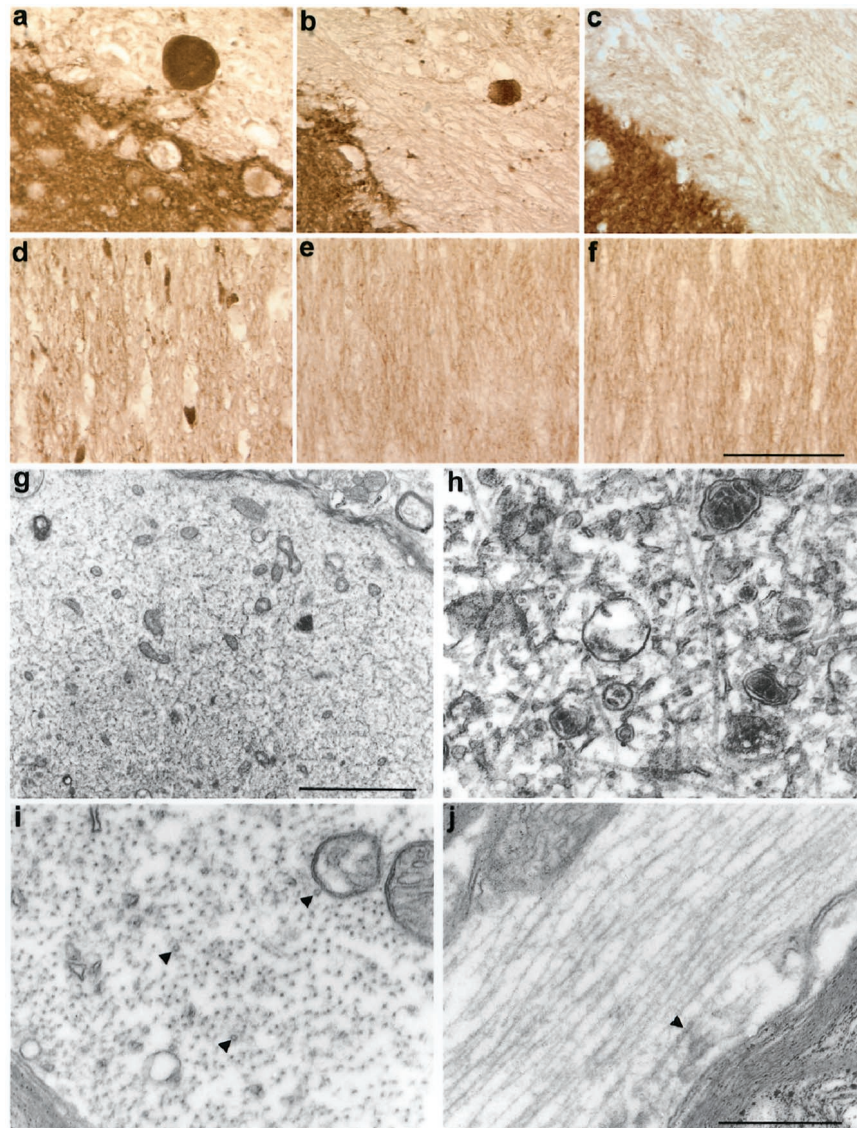


FIG. 7. Immunohistochemistry of brain and spinal cord of httau40-1 HH and httau40-1 HH \times GSK-3 β transgenic mice at 3 months and ultrastructure of axons of 7–8 months old animals. Ventral spinal cord (a) and capsula interna (b and c) stained with anti-synaptophysin. Dilated axons, immunoreactive for synaptophysin, are only detected in single httau40 transgenic mice (a and b) and are absent in double transgenic (c) and wild type (data not shown) animals. Axons in the fimbria hippocampi of httau40 transgenic mice stained with anti-synaptophysin (d). White matter of the fimbria hippocampi in httau40 \times GSK-3 β double transgenic (e) and non-transgenic (f) mice was devoid of synaptophysin immunoreactive accumulations. Bar, 50 μ m. Ultrastructural analysis of axons in ventral horn of httau40 transgenic (g and h) and httau40 \times GSK-3 β double transgenic (i and j) mice. h, in dilated axons, numerous microtubules in various orientations appear interspersed with pleomorphic and dense-cored vesicles. i and j, numerous neurofilaments but only few microtubules (arrowheads) are present in normal axons. i and j, transverse (i) and longitudinal (j) section through an axon. Bars, 2.5 μ m (g) and 500 nm (h–j).

single httau40 littermates. In this respect, our current and previous data (42) and those of others (65) are identical in that both studies fail to demonstrate protein tau filaments in transgenic mice overexpressing four repeat human protein tau. However, lampreys (69, 70) overexpressing three repeat human protein tau did form straight protein tau filaments. All data combined support the hypothesis that extra phosphorylation of protein tau is not directly responsible for the pathological aggregation into PHF, confirming recent *in vitro* data (71).

In contrast, concomitantly with hyperphosphorylation of protein tau, co-expression of GSK-3 β had a major effect on the pathology of the single httau40 transgenic mice, detailed previously (42). Indeed, practically all the pathological defects documented in the httau40 transgenic mice were nearly completely rescued by the mild overexpression of GSK-3 β . This “restoration” comprised (i) the reduction by about an order of magnitude of the number of axonal dilations in brain and spinal cord,

(ii) the reduction in axonal degeneration and muscular atrophy, and (iii) the alleviation of practically all the motoric problems. Moreover, the normalized motoric performances of GSK-3 β transgenic mice when overexpressing httau40 underscore the subtle balance between microtubule-associated and free protein tau that is required to ensure proper nerve functionality. In addition, the remarkable abundance of microtubules in a number of dilations suggest that excess protein tau binds, stabilizes existing, and/or assembles new microtubules, mimicking responses of dorsal root ganglion neurons when incubated with the microtubule-polymerizing drug paclitaxel (72). The fact that mild overexpression of GSK-3 β prevented formation of these axonal accumulations in brain and spinal cord of the double transgenic mice corroborated our observation that microtubules isolated from brain of double transgenic mice did bind less protein tau.

The mechanism of this rescue still needs to be unraveled, but

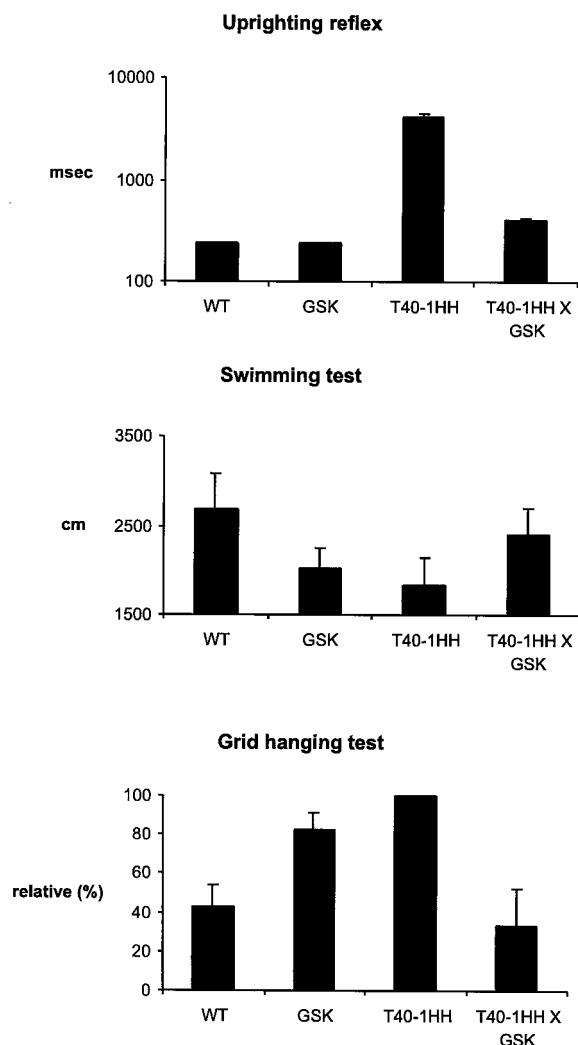


FIG. 8. Performance of single and double transgenic and wild type mice in four sensorimotor tasks. *a*, time (milliseconds, logarithmic scale) mice needed to return on four legs after being forced to lie on the back. 1HH mice were significantly slower than WT, GSK, and 1HH \times GSK littermates ($p < 0.001$). *b*, swimming speed defined as distance traveled in 2 min. GSK and 1HH mice covered shorter distances than WT mice ($p < 0.001$). However, 1HH \times GSK double transgenic and WT mice traversed the same distance ($p > 0.05$). Moreover, 1HH \times GSK mice swam faster than 1HH and GSK animals ($p < 0.05$). *c*, inverted wire grid hanging, expressed as number of mice that did not remain suspended for the entire 1-min test period, relative to the number of mice tested in each group. Significantly more GSK ($p < 0.05$) and 1HH ($p < 0.001$) mice lost hold than WT and 1HH \times GSK mice. The 1HH \times GSK double transgenic and WT mice performed equally ($p > 0.05$) well. WT, wild-type mice; 1HH, single homozygous ht40-1 transgenic mice; 1HH \times GSK, double homozygous ht40-1 heterozygous GSK-3 β transgenic mice. All mice were 2–4 months old.

the amelioration of the axonal transport, inhibited by excess MT-associated protein tau in the single ht40 transgenic mice, is the most plausible explanation. We demonstrated that the axonal dilations accumulate besides mitochondria and vesicles (42), also the synaptic marker synaptophysin, reminiscent of synaptotagmin accumulations in motor neurons of protein tau overexpressing *Drosophila* (73). This further supports the hypothesis that excess protein tau inhibited kinesin-mediated anterograde transport (74, 75) by binding to axonal microtubules (42, 55, 76–78). That hyperphosphorylation of protein tau by GSK-3 β is effectively preventing this pathological outcome is herewith demonstrated *in vivo*.

The importance of a subtle balanced phosphorylation of protein tau, as supported *in vivo* in our double transgenic mouse

model, could have therapeutic implications in the field of neurodegeneration. The success of treating manic depressive illness with lithium salts has invited propositions for its use and of drugs with similar effects to therapeutic interventions in Alzheimer's disease (79) and other tauopathies. Although lithium ions protect neurons against excitotoxicity (80), it also attenuated protein tau phosphorylation (81), most likely by inhibiting GSK-3 β (81, 82). Based on our current data, drugs that inhibit GSK-3 β could even lead to more axon damage. On the other hand, the fact that GSK-3 β is now proven to act as a protein tau kinase *in vivo* in neurons still qualifies it as a target for drug discovery.

In conclusion, we have generated and characterized double transgenic mice that overexpress constitutively active human GSK-3 β and human protein tau. Extra phosphorylation of protein tau reduces its binding capacity to microtubules, but fails to cause protein tau filament formation. The alleviation of the psychomotoric defects and the axonopathy supports *in vivo* the necessity of a balanced phosphorylation of protein tau in the functionality of central nervous system neurons. Ongoing cross-breeding of the GSK-3 β and ht40 transgenic mice with other transgenic mice that harbor other transgenes related to AD, *i.e.* amyloid precursor protein, presenilin, and apolipoproteinE4 (44, 45), will eventually provide additional information and will be suitable models for the many questions that remain open in the pathogenesis of AD.

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